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(54) Title: INCREASING STEARATE CONTENT IN SOYBEAN OIL BY EXPRESSION OF ACYL-ACP THIESTERASE

(57) Abstract

This invention relates to a method for increasing stearate as a component of total triglycerides found in soybean seed. The method generally comprises growing a soybean plant having integrated into its genome a DNA construct comprising, in the 5' to 3' direction of transcription, a promoter functional in a soybean plant seed cell, a DNA sequence encoding an acyl-ACP thioesterase protein having substantial activity on C18:0 acyl-ACP substrates, and a transcription termination region functional in a plant cell. The present invention also provides a soybean seed with about 33 weight percent or greater stearate as a component of total fatty acids found in seed triglycerides.

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INCREASING STEARATE CONTENT IN SOYBEAN OIL BY EXPRESSION OF ACYL-ACP THIESTERASE

5

INTRODUCTION

This application is a continuation-in-part of Application U.S. Serial Number 09/134,26 filed August 14, 1998.

10 Field of the Invention

The invention relates to genetic modification of plants, plant cells and seeds, particularly altering fatty acid composition.

Background

15 Soybean (*Glycine max*) is one of the highest value crops currently grown in the United States (\approx \$16 billion in 1996). Ranking close to corn (25%) and wheat (22%), soybean accounted for 19% of the United States crop acres planted in 1994. Often referred to as a “miracle crop”, soybean offers tremendous value through the oil, protein and whole soybean products. Agronomic traits, food quality traits related to oils and protein quality are all important 20 for the soybean industry.

More soybeans are grown in the United States than anywhere else in the world (2.4 billion bushels in 1996, 50% of world production). A bushel of soybean (60 pounds) is comprised of 48 pounds of protein meal and 11 pounds of oil. While protein meal is the major component in soybean, oil, lecithin, tocopherols, isoflavones, etc. are all co-products and add 25 value to the bean. Soybean oil is the major edible oil used in the world (40% of the 59.4 million metric tons in 1993). It also accounts for 70% of the 14 billion pounds of edible vegetable oil in the United States. The primary food applications where the oil is used extensively are for baking and frying (40-45%), salad and cooking oil (40-45%), margarine and shortening (15-20%) and a wide spectrum of processed foods. Development of other vegetable oils for specialty uses has

recently affected the acreage and production of soybean. The low cost and ready availability of soybean oil provide an excellent opportunity to upgrade this commodity item for specialty uses.

Food fats and oils are chemically composed of triesters of glycerol containing straight chain, normal aliphatic fatty acids, also referred to herein as triacylglycerols or triglycerides (TAG). The properties of food fats and oils are a reflection of the fatty acids contained in the TAG and their distribution on the glycerol backbone. When the melting point of the TAG is below room temperature, the TAG is referred to as an "oil". Triglycerides that melt above room temperature are referred to as "fat". Gradients between fluidity and solidity exist. Partially solidified, non-pourable triglycerides are often referred to as "plastic fats".

10 Fatty acids are organic acids having a hydrocarbon chain ranging in length from about 4 to 24 carbons. Fatty acids differ from each other in chain length, and in the presence, number and position of double bonds. In cells, fatty acids typically exist in covalently bound forms, the carboxyl portion being referred to as a fatty acyl group. The chain length and degree of saturation of these molecules is often depicted by the formula CX:Y, where "X" indicates 15 number of carbons and "Y" indicates number of double bonds.

Typically, oil derived from commercial soybean varieties is composed of approximately 11% palmitic (C16:0), 4% stearic acid (C18:0), 21% oleic acid (C18:1), 56% linoleic acid (C18:2), and 10% linolenic acid (C18:3). The fatty acid composition of soybean oil, as well as all oils, largely determines its physical and chemical properties, and thus its uses.

20 Fatty acid biosynthesis has been the subject of research efforts in a number of organisms. For reviews of fatty acid biosynthesis in plants, see Ohlrogge *et al.*, (1995) *Plant Cell*, 7:957-970, Ohlrogge *et al.*, (1997) *Annu Rev Plant Physiol Plant Mol Biol*, 48:109-136 and Sommerville *et al.* (1991) *Science*, 252:80-87.

As mentioned previously, the fatty acid composition of an oil determines its physical and 25 chemical properties, and thus its uses. Plants, especially plant species which synthesize large amounts of oils in plant seeds, for example soybean, are an important source of oils both for edible and industrial uses. Various combinations of fatty acids in the different positions in the triglyceride will alter the properties of the triglyceride. For example, if the fatty acyl groups are mostly saturated fatty acids, then the triglyceride will be solid at room temperature. In general, 30 however, vegetable oils tend to be mixtures of different triglycerides. The triglyceride oil

properties are therefore a result of the combination of triglycerides which make up the oil, which are in turn influenced by their respective fatty acid compositions.

Plant breeders have successfully modified the yield and fatty acid composition of various plant seed oils by introducing desired traits through plant crosses and selection of progeny carrying the desired trait forward. Application of this technique thus is limited to traits which are found within the same plant species. Alternatively, exposure to mutagenic agents can also introduce traits which may produce changes in the composition of a plant seed oil. However, it is important to note that Fatty Acid Synthesis (FAS) occurs in most tissues of the plant including leaf (chloroplasts) and seed tissue (proplastids). Thus, although a mutagenesis approach can sometimes result in a desired modification of the composition of a plant seed oil, it is difficult to effect a change which will not alter FAS in other tissues of the plant.

A wide range of novel vegetable oils compositions and/or improved means to obtain or manipulate fatty acid compositions, from biosynthetic or natural plant sources, are needed. Plant breeding, even with mutagenesis, cannot sufficiently meet this need and provide for the introduction of novel oil.

For example, cocoa-butter has certain desirable qualities (mouthfeel, sharp melting point, etc.) which are a function of its triglyceride composition. Cocoa-butter contains approximately 24.4% palmitate (16:0), 34.5% stearate (18:0), 39.1% oleate (18:1) and 2% linoleate (18:2). Thus, in cocoa butter, palmitate-oleate-stearate (POS) comprises almost 46% of triglyceride composition, with stearate-oleate-stearate (SOS) and palmitate-oleate-palmitate (POP) comprising the major portion of the balance at 33% and 16%, respectively, of the triglyceride composition. Other novel oils compositions of interest might include trierucin (three erucic) or a triglyceride with medium chain fatty acids in each position of the triglyceride molecule.

Plant seed oils contain fatty acids acylated at the *sn*-1, *sn*-2, and *sn*-3 positions of a glycerol backbone, referred to as a triacylglycerol (TAG). The structure of the TAG, as far as positional specificity of fatty acids, is determined by the specificity of enzymes involved in acylating the fatty acyl CoA substrates to the glycerol backbone. For example, there is a tendency for such enzymes from many temperate and tropical crop species to allow either a saturated or an unsaturated fatty acid at the *sn*-1 or the *sn*-3 position, but only an unsaturated fatty acid at the *sn*-2 in the seed TAGs. In some species such as cocoa, TAG compositions

suggest that this tendency is carried further in that there is an apparent preference for acylation of the sn-3 position with a saturated fatty acid, if the sn-1 position is esterified to a saturated fatty acid. Thus, there is a higher percentage of structured TAG of the form Sat-Un-Sat (where Sat = saturated fatty acid and Un = unsaturated fatty acid).

5 Of particular interest are triglyceride molecules in which stearate is esterified at the sn-1 and sn-3 positions of a triglyceride molecule with unsaturates in the sn-2 position particularly oleate. Vegetable oils rich in such SOS (Stearate-Oleate-Stearate) molecules share certain desirable qualities with cocoa butter yet have a degree of additional hardness when blended with other structured lipids. SOS - containing vegetable oils are currently extracted from relatively 10 expensive oilseeds from certain trees grown in tropical areas such as Sal, Shea, and Illipe trees from India, Africa, and Indonesia respectively. Cheaper and more conveniently grown sources for SOS -type vegetable oils are desirable.

In addition, vegetable oils rich in stearate fatty acid content tend to be solid at room 15 temperature. Such vegetable fats can be used directly in shortenings, margarine and other food "spread" products, obviating the need for chemical hydrogenation. Hydrogenation is a process whereby molecular hydrogen is reacted with the unsaturated fatty acid triglyceride until the desired degree of solidity is obtained. The solidity is commonly determined by the solid fat index (SFI, Official and Tentative Methods, American Oil Chemists' Society, Cd 10-57(93), Champaign, IL). Values are determined by dilatometry (expansion in volume) over a defined 20 temperature range of 50°, 70° 80°, 92° and 100° or 104°F. The hydrogenation process converts unsaturated fatty acids to partially or fully saturated fatty acids, and increases the heat and oxidative stability of the product. The iodine value (IV) measures the degree of unsaturation of a fat. Lower values indicate greater saturation. The oxidative stability may be measured by an oil stability index (Official and Tentative Methods, American Oil Chemists' Society, Cd 1b-87, 25 Champaign, IL) and active oxygen method (AOM, Official and Tentative Methods, American Oil Chemists' Society, Cd 12h-92, Champaign, IL). The cost and any other factors associated with chemical hydrogenation, such as the production of trans fatty acids, can be avoided if the vegetable oil is engineered to be stearate rich in the plant seed.

Moreover, some plant tissues use 18 carbon fatty acids as precursors to make other 30 compounds. These include saturated long chain fatty acids longer than 18 carbons in length.

Since very little stearate typically accumulates in soybean plants, it may be necessary to increase stearate accumulation if one wants to increase production of compounds which depend upon supply of stearate fatty acids for synthesis.

The fatty acid composition of soybean oil described above is often considered less than 5 optimal in terms of oil functionality. While the limitations of the fatty acid composition may be partly overcome by chemical hydrogenation, the trans fatty acids produced as a result of the hydrogenation process are Sat-Un-Satpected of having unfavorable health effects (Mensink, *et al.* (1990) *N. Eng. J. Med.* 323:439-445).

Through the efforts of traditional plant breeding techniques, the fatty acid composition of 10 soybeans has been improved. For example, using mutagenesis, plant breeders have been able to increase the amount of stearate (C18:0) produced in the soybean oil. In such high stearate lines, designated as A6 (ATCC Accession No. 97392, Hammond and Fehr, (1983) *Crop Science* 23:192), stearate levels of up to about 25 weight percent of the total fatty acid composition have been achieved. Such high stearate containing lines have been further bred with mutant soybean 15 lines containing elevated levels of palmitate (16:0). Soybean lines containing the elevated stearate levels produced by mutagenesis demonstrate a negative correlation of increased stearate content and seed yield (Hartmann, *et al.* (1997) *Crop Science* 37:124-127). Attempts to further increase the stearate content and/or improve the seed yield of such increased stearate lines by breeding have thusfar proven unsuccessful.

20 List, *et al.* ((1996) *J. Am. Oil. Chem. Soc.* 73:729-732) describes the use of genetically modified soybean oils in margarine formulations. High stearate oil from soybean variety A6 was found to have an insufficient solid fat index at 24.7°C and higher temperatures to make margarine. The soybean oil was blended with cottonseed or soybean hardstocks to afford mixtures with sufficient solids content for formulation into margarine.

25 While soybean based products are a major food source, improvements to the nutritional and commercial quality of this product could add further value to soybean based products. Alteration of the soybean oil content and composition could result in products of higher nutritional content and greater stability. The need for industrial hydrogenation of polyunsaturated oil for food applications could be reduced by the preparation of soybean oil with 30 increased concentrations of stearate.

SUMMARY OF THE INVENTION

The present invention is directed to methods for producing soybean oil having high levels of stearate (C18:0). The method of producing a soybean oil containing increased levels of stearate comprises expression of an acyl-ACP thioesterase capable of producing C18:0 in the seed tissue of the soybean. In particular, the acyl-ACP thioesterase has substantial activity toward 18:0 acyl-ACP substrates, and preferably has little or no activity towards 16:0 acyl-ACP substrates.

The method generally comprises growing a soybean plant containing a construct comprising as operably linked components in the 5' to 3' direction of transcription, a transcription initiation region functional in a seed tissue and a DNA encoding an acyl-ACP thioesterase with substantial activity towards 18:0 acyl-ACP substrates and a transcription termination sequence.

The stearate content of the soybean oil preferably comprises greater than about 20%, more preferably greater than about 33% of the fatty acid moieties in the oil. The oil of the present invention may be used as a blending source to make a blended oil product, or it may also be used in the preparation of food.

In another embodiment of the present invention, a soybean oil having an increased saturated fatty acid composition is provided. Soybean oils with saturated fatty acid compositions of greater than 50 weight percent are exemplified herein.

In yet another embodiment of the instant invention, the novel soybean oil, comprising the increased total saturated fatty acid compositions, provides a novel source of structured TAG of the Sat-Un-Sat (saturated-unsaturated-saturated) form.

The present invention further provides food products and methods for their preparation from a novel soybean [*Glycine max*] seed with increased levels of stearic and oleic acids, and decreased levels of linoleic and linolenic acids under normal growing conditions. The novel soybean seed is produced by a soybean plant obtained from cross pollination of a high stearate plant with a low linolenate plant. There are multiple advantages of a soybean seed with modified fatty acid content. The soybean oil has increased levels of stearic acid, normal levels of

palmitic and oleic acids, and decreased levels of linoleic and linolenic acids relative to common soybean oil. Preferably, the soybean oil has a stearic acid composition of above about 15%, a linoleic acid composition below about 45%, and a linolenic acid composition below about 6%.

Oil extracted from the soybean seeds possess increased stability and superior cooking

5 characteristics than does oil extracted from standard soybean seeds. The oil has higher levels of solids than does common soybean oil, making it a more preferred material for the preparation of food products such as margarine, tofu, soy flour, soymilk, and shortening. Interesterification of the oil can further enhance the amount of solids present, and the oil's utility in the preparation of food products. Food products prepared from modified soybeans display creamier textures than
10 do food products prepared from common soybeans. While common and high stearate soybean oils require the addition of hardstocks for the formation of margarines and other soy based products, the present oil may be used without the addition of adjuvants.

The novel soybean oil as well as the soybean seed containing the novel oil finds use in many applications.

15

DESCRIPTION OF THE FIGURES

Figure 1. Nucleic acid and translated amino acid sequence of a mangosteen FatA-type acyl-ACP thioesterase clone (*Garm FatA1*) is provided. *Garm FatA1* demonstrates primary
20 thioesterase activity on 18:1 acyl-ACP substrate, but also demonstrates substantial activity on 18:0 substrate (approximately 10-20% of 18:1 activity), as well as little or no activity on 16:0 substrates.

DETAILED DESCRIPTION OF THE INVENTION

25

In accordance with the subject invention, constructs and methods are provided for the production of soybean plants with an increased level of stearate (C18:0), as a percentage of the total fatty acids, in the seed oil. The methods for producing such soybean plants comprise transforming a soybean plant with expression constructs comprising a promoter sequence
30 functional in a plant seed operably linked to a DNA sequence encoding a plant acyl-ACP

thioesterase having substantial activity toward 18:0-ACP substrates, preferably those with little or no activity toward 16:0-ACP substrates (hereinafter referred to as stearoyl-ACP thioesterase), and a transcription termination sequence. The expression constructs provide an increase in the levels of stearate fatty acids in the seed oil of the transformed soybean plants.

5 As described in more detail in the examples that follow, an acyl-ACP thioesterase coding sequence from mangosteen (*Garcinia mangostana*), Garm *FatA1* (Hawkins and Kridl (1998) *Plant Journal* 13(6):743-752; and PCT Patent Application WO 96/36719, the entireties of which are incorporated herein by reference) is used in expression constructs to generate transgenic soybean plants with increased production of the stearoyl-ACP thioesterase in host cells. In 10 particular the constructs are used to direct the expression of the *Garm FatA1* thioesterase in plant seed cells for modification of triacylglycerol (TAG) fatty acid composition to provide increased levels of C18:0 fatty acyl groups. Furthermore, the constructs of the present invention may find use in plant genetic engineering applications in conjunction with plants containing elevated levels of C18:0 (stearate) fatty acids. Such plants may be obtained by antisense gene regulation 15 of stearoyl-ACP desaturase using methods described by Knutzon *et al.* (*Proc. Nat. Acad. Sci.* (1992) 89:2624-2628), and may also be obtained by co-suppression using sense expression constructs of the stearoyl-ACP desaturase gene, or by conventional mutation and plant breeding programs. In addition, the constructs and methods for increasing stearate in soybean seed may also find use in plant genetic engineering applications in conjunction with plants containing 20 elevated levels of oleate (C18:1) and/or decreased levels of linoleate (C18:2) fatty acids and/or linolenate (18:3). Such plants with elevated levels of oleate and/or with decreased levels of linoleate and/or linolenate may be obtained through genetic engineering, or by conventional mutation and plant breeding programs.

A plant acyl-ACP thioesterase DNA sequence useful for the preparation of expression 25 constructs for the alteration of stearate levels as described herein encodes for amino acids, in the form of a protein, polypeptide or peptide fragment, which amino acids demonstrate substantial activity on 18:0 acyl-ACP substrates and little or no activity on 16:0-ACP to form 18:0 free fatty acid (i.e., stearate) under plant enzyme reactive conditions. By "enzyme reactive conditions" is meant that any necessary conditions are available in an environment (i.e., such factors as 30 temperature, pH, lack of inhibiting substances) which will permit the enzyme to function.

DNA sequences encoding for acyl-ACP thioesterase enzymes with substantial activity on 18:0 acyl-ACP substrates and little or no activity on 16:0-ACP to form 18:0 free fatty acid (i.e., stearate) are known in the art and are described in Hawkins and Kridl (1998) *supra*, and PCT Patent Application WO 96/36719. The *Garm* FatA1 DNA sequence described therein and used 5 herein demonstrates preferential activity on C18:1 acyl-ACP substrate, and also demonstrates substantial activity (approximately 25% of the 18:1 activity) on C18:0 acyl-ACP substrates. Only a small increase in C16:0 activity over activity in control cells is observed, and the 16:0 activity represents only approximately 3% of the 18:1 activity.

In preparing the expression constructs, the various DNA fragments may be manipulated, 10 so as to provide for the DNA sequences in the proper orientation and, as appropriate in the proper reading frame. Towards this end, adapters or linkers may be employed to join the DNA fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous DNA, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resection, ligation, or the like may be 15 employed, where insertions, deletions or substitutions, *e.g.* transitions and transversions, may be involved.

For the most part, the constructs will involve regulatory regions functional in plants 20 which provide for modified production of plant stearoyl-ACP thioesterase, and modification of the fatty acid composition. The open reading frame, coding for the plant stearoyl-ACP thioesterase or functional fragment thereof will be joined at its 5' end to a transcription initiation regulatory region such as the wild-type sequence naturally found 5' upstream to the thioesterase structural gene, or to a heterologous regulatory region from a gene naturally expressed in plant tissues. Examples of useful plant regulatory gene regions include those from T-DNA genes, such as nopaline or octopine synthase, plant virus genes, such as CaMV 35S, or from native 25 plant genes.

For such applications when 5' upstream non-coding regions are obtained from other genes regulated during seed maturation, those preferentially expressed in plant embryo tissue, such as ACP, napin and β -conglycinin 7S subunit transcription initiation control regions, as well as the *Lesquerella* hydroxylase promoter (described in Broun, *et al.* (1998) *Plant Journal* 30 13(2):201-210 and in U.S. Patent Application Serial Number 08/898,038) and the stearoyl-ACP

desaturase promoter (Slocombe, *et al.* (1994) *Plant Physiol.* 104:1167-1176), are desired. Such "seed-specific promoters" may be obtained and used in accordance with the teachings of USPN 5,420,034 having a title "Seed-Specific Transcriptional Regulation" and in Chen *et al.*, (1986), *Proc. Natl. Acad. Sci.*, 83:8560-8564. Transcription initiation regions which are preferentially expressed in seed tissue, i.e., which are undetectable in other plant parts, are considered
5 desirable for fatty acid modifications in order to minimize any disruptive or adverse effects of the gene product.

Regulatory transcript termination regions may be provided in DNA constructs of this invention as well. Transcript termination regions may be provided by the DNA sequence
10 encoding the plant stearoyl-ACP thioesterase or a convenient transcription termination region derived from a different gene source, for example, the transcript termination region which is naturally associated with the transcript initiation region. The skilled artisan will recognize that any convenient transcript termination region which is capable of terminating transcription in a plant cell may be employed in the constructs of the present invention. As described herein,
15 transcription termination sequences derived from DNA sequences preferentially expressed in plant seed cells are employed in the expression constructs of the present invention.

The method of transformation is not critical to the instant invention; various methods of plant transformation are currently available. As newer methods are available to transform crops, they may be directly applied hereunder. For example, many plant species naturally susceptible
20 to *Agrobacterium* infection may be successfully transformed via tripartite or binary vector methods of *Agrobacterium*-mediated transformation. In addition, techniques of microinjection, DNA particle bombardment, and electroporation have been developed which allow for the transformation of various monocot and dicot plant species.

In developing the DNA construct, the various components of the construct or fragments
25 thereof will normally be inserted into a convenient cloning vector which is capable of replication in a bacterial host, e.g., *E. coli*. Numerous vectors exist that have been described in the literature. After each cloning, the plasmid may be isolated and subjected to further manipulation, such as restriction, insertion of new fragments, ligation, deletion, insertion, resection, etc., so as to tailor the components of the desired sequence. Once the construct has

been completed, it may then be transferred to an appropriate vector for further manipulation in accordance with the manner of transformation of the host cell.

Normally, included with the DNA construct will be a structural gene having the necessary regulatory regions for expression in a host and providing for selection of transformant 5 cells. The gene may provide for resistance to a cytotoxic agent, e.g. antibiotic, heavy metal, toxin, etc., complementation providing prototrophy to an auxotrophic host, viral immunity or the like. Depending upon the number of different host species in which the expression construct or components thereof are introduced, one or more markers may be employed, where different conditions for selection are used for the different hosts. A number of markers have been 10 developed for use for selection of transformed plant cells, such as those which provide resistance to various antibiotics, herbicides, or the like. The particular marker employed is not essential to this invention, one or another marker being preferred depending on the particular host and the manner of construction.

As mentioned above, the manner in which the DNA construct is introduced into the plant 15 host is not critical to this invention. Any method which provides for efficient transformation may be employed. Various methods for plant cell transformation include the use of Ti- or Ri- plasmids, microinjection, electroporation, DNA particle bombardment, liposome fusion, or the like. In many instances, it will be desirable to have the construct bordered on one or both sides by T-DNA, particularly having the left and right borders, more particularly the right border. 20 This is particularly useful when the construct uses *A. tumefaciens* or *A. rhizogenes* as a mode for transformation, although the T-DNA borders may find use with other modes of transformation.

Various methods of transforming cells of soybean have been previously described. Examples of soybean transformation methods have been described, for example, by Christou *et* 25 *al.* U.S. Patent Number 5,015,580 and by Hinchee *et al.* U.S. Patent Number 5,416,011, the entireties of which are incorporated herein by reference.

Once a transgenic plant is obtained which is capable of producing seed having a modified fatty acid composition, traditional plant breeding techniques, including methods of mutagenesis, may be employed to further manipulate the fatty acid composition. Alternatively, 30 additional foreign fatty acid modifying DNA sequence may be introduced via genetic engineering to further manipulate the fatty acid composition.

One may choose to provide for the transcription or transcription and translation of one or more other sequences of interest in concert with the expression of a plant stearoyl-ACP thioesterase in a plant host cell. In particular, the reduced expression of stearoyl-ACP desaturase in combination with expression of a plant stearoyl-ACP thioesterase may be preferred in some 5 applications.

When one wishes to provide a plant transformed for the combined effect of more than one nucleic acid sequence of interest, typically a separate nucleic acid construct will be provided for each. The constructs, as described above contain transcriptional or transcriptional and translational regulatory control regions. The constructs may be introduced into the host cells by 10 the same or different methods, including the introduction of such a trait by the inclusion of two transcription cassettes in a single transformation vector, the simultaneous transformation of two expression constructs, retransformation using plant tissue expressing one construct with an expression construct for the second gene, or by crossing transgenic plants via traditional plant breeding methods, so long as the resulting product is a plant having both characteristics 15 integrated into its genome.

By decreasing the amount of stearoyl-ACP desaturase, an increased percentage of saturated fatty acids is provided. Using anti-sense, transswitch, ribozyme or some other stearoyl-ACP desaturase reducing technology, a decrease in the amount of stearoyl-ACP desaturase available to the plant cell is produced, resulting in a higher percentage of saturates such as one or 20 more of stearate (C18:0), arachidate (C20:0), behenate (C22:0) and lignocerate (C24:0). In rapeseed reduced stearoyl-ACP desaturase results in increased stearate levels and total saturates (Knutzon *et al.* (1992) *Proc. Nat. Acad. Sci* 89:2264-2628).

Of special interest is the production of triglycerides having increased levels of stearate. In addition, the production of a variety of ranges of stearate is desired. Thus, plant cells having 25 lower and higher levels of stearate fatty acids are contemplated. For example, fatty acid compositions, including oils, having a 10% level of stearate as well as compositions designed to have up to an approximate 60% level of stearate or other such modified fatty acid(s) composition are contemplated.

As described in more detail in the examples that follow, constructs are prepared to direct 30 the expression of a stearoyl-ACP thioesterase in plant seed tissue. Such expression constructs

allow for the increase in 18:0 levels in oils obtained from the seeds of transformed soybean plants.

Increases in the levels of stearate in soybeans transformed to express *Garm* FatA1 range from 4 fold to approximately 13 fold over the levels obtained in seeds from nontransformed control

5 plants. Additionally, by decreasing the amount of stearoyl-ACP desaturase available to the plant FAS complex in conjunction with an increase of the amount of stearoyl-ACP thioesterase available, a more marked increased percentage of stearate may be obtained. By manipulation of various aspects of the DNA constructs (e.g., choice of promoters, number of copies, etc.) and traditional breeding methods, one skilled in the art may achieve even greater levels of stearate.

10 By expression of a plant stearoyl-ACP thioesterase in seed tissue or a decrease in the expression of stearoyl-ACP desaturase or a combination of both, an increased percentage of stearate can be achieved in soybean. In addition, modified thioesterase encoding DNA sequences may find use for increasing stearate levels in seed tissue. Such modified thioesterase sequences may be obtained as described in PCT Patent Application WO 96/36719.

15 Surprisingly, in the oil of seeds from T2 soybean lines transformed to express the *Garm* FatA1 DNA sequence from the β -conglycinin 7S subunit promoter, stearate levels of up to 53 percent as a percentage of the total fatty acid composition are obtained. In addition, in the oil of the initial transformed lines expressing *Garm* FatA1 DNA sequence from the β -conglycinin 7S subunit promoter increases in the levels of stearate obtained from individual seeds range from 20 about 14 weight percent up to about 53 weight percent. Furthermore, transgenic soybean plants expressing the *Garm* FatA1 DNA sequence from the napin derived promoter accumulate increased levels of stearate ranging from approximately 20 weight percent up to approximately 45 weight percent in individual seeds of T2 soybean lines. Stearate levels obtained from the oil of individual seeds of nontransformed control soybeans range from approximately 4 weight percent to approximately 6 weight percent. Preferred oil compositions for many applications include 33 weight percent or greater stearate fatty acids as a component of the soybean oil.

25 In addition, transformed soybean lines containing increased stearate levels of the present invention also demonstrate an increase in the total levels of saturated fatty acids. Transformed soybean lines containing elevated stearate levels also contain increased levels of Arachidic acid (30 20:0) and Behenic acid (22:0). Increases in 20:0 range from about 3 fold to about 11 fold over

the levels of 20:0 obtained from the seed oil of nontransgenic control soybean lines. Increases in 22:0 range from about 2 fold to about 5 fold over the levels of 22:0 obtained from the seed oil of nontransgenic control soybean lines.

Thus, in soybean lines transformed to express a stearoyl- ACP thioesterase in the seed 5 tissue, total saturated fatty acids (16:0, 18:0, 20:0 and 22:0) comprise at least 30 percent of the total fatty acids as a percentage of weight, preferably above 50 weight percent. In some cases, total saturated fatty acid levels of above about 65 weight percent may be obtained.

The novel soybean oil compositions of the present invention comprise increased total 10 saturated fatty acids and provide a novel source of structured TAG of the Sat-Un-Sat form. For oil compositions having greater than about 33 weight percent stearate the Sat-Un-Sat form of 15 TAG may comprise 25 percent or greater of the total TAG composition as a stearate-unsaturated-stearate form of TAG. It is apparent that by utilizing a high oleic acid soybean line that one may produce a soybean oil with a high proportion of a stearate-oleate-stearate form of TAG. An example of such high oleic acid soybean oil is described in PCT Application WO 97/40698.

The present invention further provides food products and methods for their preparation 15 from a novel soybean [*Glycine max*] seed with increased levels of stearic and oleic acids, and decreased levels of linoleic and linolenic acids under normal growing conditions. The novel soybean seed is produced by a soybean plant obtained from cross pollination of a high stearate plant with a low linolenate plant. There are multiple advantages of a soybean seed with 20 modified fatty acid content. The soybean oil has increased levels of stearic acid, normal levels of palmitic and oleic acids, and decreased levels of linoleic and linolenic acids relative to common soybean oil. Preferably, the soybean oil has a stearic acid composition of above about 15%, a linoleic acid composition below about 45%, and a linolenic acid composition below about 6%.

Oil extracted from the soybean seeds possess increased stability and superior cooking 25 characteristics than does oil extracted from standard soybean seeds. The oil has higher levels of solids than does common soybean oil, making it a more preferred material for the preparation of food products such as margarine, tofu, soy flour, soymilk, and shortening. Interesterification of the oil can further enhance the amount of solids present, and the oil's utility in the preparation of food products. Food products prepared from modified soybeans display creamier textures than 30 do food products prepared from common soybeans. While common and high stearate soybean

oils require the addition of hardstocks for the formation of margarines and other soy based products, the present oil may be used without the addition of adjuvants.

The soybean oil compositions of the present invention containing novel fatty acid compositions may find use in a number of applications, without the need for chemical 5 modifications prior to use as described herein, or as described in PCT application titled "Food Products Containing Structured Triglycerides", PCT/US97/06037, the entirety of which is incorporated herein by reference. The soybean oil of the present invention may find use in the preparation of foods to facilitate cooking or heating applications.

The soybean oils produced by the methods of the present invention may be used in the 10 formation of emulsions comprising water and soybean oil. The soybean oil may be treated by interesterification prior to the formation of an emulsion. As used herein, interesterification refers to the process of rearranging the glyceride structure of fats. Interesterification is accomplished by a chemical reaction in which fatty acids are rearranged on the glycerol molecule without modification of the fatty acids themselves. An emulsion may preferably 15 comprise between about 70% and about 90% by volume soybean oil, and between 10% and about 30% by volume water. The aqueous emulsion may further be defined as margarine. As used herein, the term "margarine" refers to an edible emulsion comprising oil and water that is both solid and spreadable at 25°C.

Alternatively, the soybean seeds containing the modified fatty acid compositions of the 20 present invention, may be used to prepare soymilk. Soymilk may be prepared by the steps of selecting soybean seeds, contacting the seeds with water to form a mixture, heating the mixture, grinding the mixture, and removing the solids to form soymilk. Removal of solids may be accomplished by methods including, but not limited to, filtration, sedimentation and centrifugation. The heating step may comprise heating the mixture to any temperature suitable 25 for the formation of tofu, preferably to a temperature sufficient to inactivate the trypsin inhibitor in the liquid at least about 80% as compared to the trypsin inhibitor activity prior to heating, and most preferably to a temperature between about 90° and about 100°C. Trypsin inhibitor activity may be conveniently assayed using colorimetric method described in Liu and Markakis ((1989), *Cereal Chem* 66(5):415-422).

Soybean seeds containing the novel soybean oil compositions of the present invention may find use in the preparation of tofu. Tofu may be prepared by the steps of selecting soybean seeds containing soybean oil having oil compositions of the present invention, contacting the seeds with water to form a mixture, heating the mixture, grinding the mixture, removing the 5 solids to form a filtrate, adding a coagulant, and cooling the filtrate to form tofu. The coagulant used may be, but is not limited, to glucono- δ -lactone, lemon juice, sea salt, calcium sulfate or magnesium chloride. Removal of solids may be accomplished by methods including, but not limited to filtration, sedimentation, and centrifugation. The heating step may comprise heating the mixture to any temperature suitable for the formation of tofu, preferably to a temperature 10 sufficient to inactivate the trypsin inhibitor in the liquid at least about 80% as compared to the trypsin inhibitor activity prior to heating, and most preferably to a temperature between about 90° and about 100°C. Trypsin inhibitor activity may be conveniently assayed using colorimetric method described in Liu and Markakis ((1989), *Cereal Chem* 66(5):415-422). The cooling step may comprise cooling the filtrate to any temperature suitable for the formation of tofu, and more 15 preferably to between about 0°C and about 25°C.

Soy flour may also be prepared from soybean seeds containing the oil compositions of the present invention. A method for preparation of soy flour comprises the steps of selecting soybean seeds and grinding the seeds to produce soy flour. Preferably, the soybean seeds contain soybean oil having increased levels of stearate. The grinding step may be performed by 20 any means suitable for the production of soy flour, including, but not limited to, grinding with wheels, mortar and pestle, plates and blades.

The soybean oil of the present invention may be further used to prepare shortenings. As used herein, the term "shortening" refers to fats, usually plastic in nature, that provide functional effects related to structure, texture and the eating qualities of a variety of food products. The 25 shortening prepared based on the soybean oil of the present invention may also include the incorporation of emulsifiers or surfactants (selected from 21 C.F.R. 172), such emulsifiers used to provide additional functional effects in the final food product. The shortening based on the soybean oil of the present invention may also include a variety of hardstocks (fully hydrogenated triglycerides sourced from a variety of common food oils) that could be used to modify or 30 augment the SFC of the final product and, hence, the physical properties and functionality of the

final blend. The plasticity and crystal structure of the final shortening composition based on the soybean oil of the present invention, whether emulsified or not, may be further modified through the process of controlled crystallization and specific gravity reduction known as votation. In this process, the molten shortening described variously above is fed through a scraped-surface heat 5 exchanger, and crystallized in a directed manner-usually in the most functional crystalline form. During this process, a gas is usually whipped into the solidifying product to adjust the specific gravity of the shortening product, and hence its plasticity. This is done, primarily, to enhance the handling characteristics of the final shortening and to allow it to be better incorporated into a variety of food product systems. The emulsifier may generally be any material suitable for the 10 preparation of shortening, preferably an emulsifier approved as a food additive per 21 C.F.R. § 172, and more preferably is a monoglyceride. The use of the novel soybean oil of the present invention may allow for a reduced level of emulsifier addition to achieve the same functional effects as would be required in a standard soybean oil based shortening arrived at through hydrogenation to attain equal solids.

15 The soybean seeds, oil, and products therefrom may also find use in a number of additional applications known to the art, including the use in various animal feed applications.

The invention now being generally described, it will be more readily understood by reference to the following examples which are included for purposes of illustration only and are not intended to limit the present invention.

20

EXAMPLES

Example 1: Plant Expression Vector Construction

Plant vectors are constructed to control the expression of a member of the FatA class of 25 acyl-ACP thioesterases from *Garcinia mangostana* (*Garm FatA1*, Hawkins and Kridl (1998) *supra*, and PCT Patent Application WO 96/36719) in seeds of soybean utilizing different seed enhanced promoters.

A plant transformation construct, pWRG5374, is prepared to express *Garm FatA1* in the embryo tissue of the soybean seed utilizing the napin promoter. A DNA fragment containing the 30 napin 5'/*Garm FatA1*/napin 3', described in Hawkins and Kridl (1998) *supra*, is cloned into a

vector containing the selectable marker β -glucuronidase (GUS, Jefferson *et al.*, *Proc. Natl. Acad. Sci.* (1986) 83:8447-8451) driven by the CAMV 35S (Gardner, *et al.* (1981) *Nucleic Acids Res.* 9:2871-2888) promoter. The GUS gene contains an untranslated leader sequence derived from a soybean *ribulose-bis-phosphate carboxylase* (RuBisCo) small subunit, (Grandbastien, *et al.* (1986) *Plant Mol. Biol.* 7:451-466), ssuL and a translational termination sequence derived from the soybean RuBisCo, (Berry-Lowe (1982) *Jour. Mol. Appl. Genet.* 1:483-498), SpA. Examples of vectors utilizing a GUS selectable marker are described in European Patent 0 301 749 B1, the entirety of which is incorporated herein by reference. The resulting expression construct, pWRG5374, contains the napin 5'/*Garm* FatA1/napin 3' sequences as well as the 35S-ssuL/GUS/SpA 3' for transgenic selection by indigo blue staining.

The soybean transformation construct, pWRG5378, containing the *Garm* FatA1 coding sequence expressed from the β -conglycinin 7S subunit promoter was prepared as follows. The *Garm* FatA1 coding sequence and napin 3' poly-A termination sequences were obtained from plasmid pCGN5253 (described in Hawkins and Kridl (1998) *supra*). A soybean expression plasmid pWRG5375 was constructed by insertion of the *Garm* FatA1 coding and napin 3' sequences downstream of a heterologous promoter from the soybean α' subunit of β -conglycinin (soy 7s, (Chen *et al.*, (1986), *Proc. Natl. Acad. Sci.*, 83:8560-8564)). A 941bp BamHI-XbaI fragment containing the soy 7s promoter was ligated with a 5186bp fragment from plasmid pCGN5253 produced by partial digestion with KpnI and complete digestion with SalI. Additionally, an 8bp BamHI-KpnI adapter having the DNA sequence 5'-GATCGTAC-3' was used to fuse the BamHI site from the soy 7s promoter fragment with the KpnI site from pCGN5253. The resulting plasmid was named pWRG5375. A 3477bp SacII fragment from plasmid pWRG5375 containing the soy 7s/*Garm* FatA1/napin 3' was ligated to a 6135bp fragment containing the β -glucuronidase (GUS) marker cassette (described above) for selection of transgenic soybean plants. The soy 7s/*Garm* FatA1/napin 3' cassette was inserted such that the transcription of the GUS gene was in the same direction as that of the *Garm* FatA1 coding sequence. The resulting 8329bp plasmid was designated as pWRG5378.

Example 2: Soybean Transformation with *Garm* FatA1 Constructs

Plasmids pWRG5374 and pWRG5378 were digested with NotI and linearized fragments containing both the chimeric *Garm* *FatA1* coding sequence and GUS expression cassettes were purified by HPLC. The linear DNA fragments were stably introduced into soybean (Asgrow variety A5403) by the method of McCabe, et.al. (1988) Bio/Technology 6:923-926.

5 Transformed soybean plants are identified by indigo blue staining of seed tissue with 1 mM X-Gluc (Clontech), 0.1M NaPO₄ (pH 7.0), 0.5 mM potassium ferrocyanide.

Example 3: Fatty Acid Compositional Analysis

Fatty acid compositions were analyzed from seed of soybean lines transformed with 10 pWRG5374 or pWRG5378. One to five seeds of each of the transgenic and control soybean lines were ground individually using a tissue homogenizer (Pro Scientific) for oil extraction. Oil from ground soybean seed was extracted overnight in 1.5 ml heptane containing 15 triheptadecanoic (0.50 mg/ml). Aliquots of 200 μ l of the extracted oil was derivatized to methyl esters with the addition of 500 μ l sodium methoxide in absolute methanol. The derivatization 20 reaction was allowed to progress for 20 minutes at 50°C. The reaction was stopped by the simultaneous addition of 500 μ l 10% (w/v) sodium chloride and 400 μ l heptane. The resulting fatty acid methyl esters extracted in hexane were resolved by gas chromatography (GC) on a Hewlett Packard model 6890 GC. The GC was fitted with a Supelcowax 250 column (30 m, 0.25 mm id, 0.25 micron film thickness)(Supelco, Bellefonte, PA). Column temperature was 25 175°C at injection and the temperature programmed from 175°C to 245°C to 175°C at 40°C/min. Injector and detector temperatures were 250°C and 270°C, respectively.

The results of the fatty acid compositional analysis from seed oil of the initial transformed 5374 soybean lines is provided in Table 1. Averages are provided where oil 25 compositional analysis was performed on more than one seed from the initial transformant. In seed of transgenic soybean plants expressing *Garm* *FatA1* from the napin promoter, stearate (C18:0) levels were significantly increased over the levels obtained from the seed oil of nontransformed control plants. The increase in stearate is primarily at the expense of oleate, and to a lesser degree linoleate and palmitic all of which were decreased in the transgenic lines. In addition, increases in all saturates examined greater than C18:0 were observed.

Table 1

STRAIN ID	GUS	%16:0	%18:0	%18:1	%18:2	%18:3	%20:0	%22:0
5374-A5403-3	+	6.71	24.88	14.53	43.73	6.99	1.84	1.03
5374-A5403-3	+	6.62	26.52	11.9	44.89	7.15	1.8	0.9
5374-A5403-3	+	7.59	22.99	13.17	45.32	8.19	1.63	0.84
5374-A5403-3	+	7.28	23.1	13	43.65	9.74	1.88	1.13
5374-A5403-3	+	7.39	26.54	8.4	41.95	12.38	1.9	1.07
AVERAGE		7.12	24.81	12.2	43.91	8.89	1.81	0.99
5374-A5403-4	+	7.38	20.81	16.45	46.61	5.99	1.57	0.87
5374-A5403-4	+	7.96	18.28	14.69	47.93	8.29	1.54	0.96
5374-A5403-4	+	10.02	9.64	22.78	49.41	6.02	0.91	0.79
5374-A5403-4	+	9.24	12.54	23.06	45.97	5.9	1.17	0.8
5374-A5403-4	+	7.41	20.07	13.42	45.91	10.32	1.55	0.97
AVERAGE		8.40	16.27	18.08	47.17	7.29	1.35	0.88
5374-A5403-14	+	7.96	38.39	7.82	37.34	6.23	2.41	1.07
5374-A5403-35	+	9.24	33.53	11.14	38.15	7.54	1.97	0.9
5374-A5403-36	+	8.18	19.37	13.92	47.05	8.47	1.6	1.06
5374-A5403-36	+	7.5	19.99	13.49	47.15	8.99	1.59	0.95
5374-A5403-36	+	7.05	23.44	10.54	46.19	9.64	1.82	0.99
5374-A5403-36	+	7.83	20.06	13.53	47.27	8.45	1.57	0.94
5374-A5403-36	+	7.49	22.9	11.81	46.14	8.54	1.72	1.03
AVERAGE		7.61	21.15	12.66	46.76	8.82	1.66	0.99
5374-A5403-172	+	7.92	15.53	29.9	38.2	5.8	1.36	0.89
5374-A5403-172	+	7.37	22.45	16.63	44.3	6.58	1.56	0.77
5374-A5403-172	+	8.74	14.38	20.17	47.33	6.94	1.17	0.84
AVERAGE		8.01	17.45	22.23	43.28	6.44	1.36	0.83
Control								
A5403	-	11.62	4.3	24.26	49.84	7.47	0.48	0.57
A5403	-	12.32	4.24	21.93	52.32	7.49	0.44	0.46
A5403	-	12.64	4.25	20.49	53.42	7.81	0.43	0.51
A5403	-	12.17	4.22	21.56	52.48	8.15	0.44	0.51
A5403	-	11.68	4.32	25.68	49.67	7.06	0.48	0.54
AVERAGE		12.09	4.27	22.78	51.55	7.60	0.45	0.52

Selected T2 lines also show the trends of increased stearate, and decreased palmitate, oleate and linoleate levels in the seed oil (Table 2). Furthermore, in seed of T2 5374 soybean 5 lines (T3 seed), stearate levels as high as approximately 45% of the fatty acid methyl esters are observed. These levels are increased from approximately 34% in the T1 generation. While null progeny which do not contain the *Garm FatA1* transgene contain approximately 4.5% of the fatty acid methyl esters as stearate.

Table 2

STRAIN ID	GUS	16:0	18:0	18:1	18:2	18:3	20:0	22:0
5374-A5403-3-417	+	6.57	37.18	7.39	35.38	9.36	2.68	1.2
5374-A5403-3-417	+	6.52	38.66	8.26	33.82	8.43	2.78	1.29
5374-A5403-3-417	+	6.23	39.26	7.26	35.11	7.84	2.8	1.26
5374-A5403-3-417	+	6.75	33.55	8.91	37.69	9.13	2.51	1.19
5374-A5403-3-417	+	6.18	42.21	5.88	33.36	7.99	2.94	1.23
average		6.45	38.17	7.54	35.07	8.55	2.74	1.23
5374-A5403-35-483	+	5.78	45.64	6.3	31.86	6.15	2.95	1.16
5374-A5403-35-483	+	5.82	38.21	7.83	37.06	7.22	2.54	1.12
5374-A5403-35-483	+	5.84	38.37	7.44	37.91	6.43	2.59	1.22
5374-A5403-35-483	+	5.74	41.56	6.31	35.4	6.98	2.71	1.12
5374-A5403-35-483	+	5.58	40.35	7.06	36.91	6.11	2.63	1.16
average		5.75	40.83	6.99	35.83	6.58	2.68	1.16
5374-A5403-172-401	+	6.73	23.12	15.02	46.04	6.05	1.77	0.97
5374-A5403-172-401	+	6.92	21.96	14.85	46.47	6.7	1.78	1.02
5374-A5403-172-401	+	6.49	24.15	14.11	45.74	6.46	1.83	0.96
5374-A5403-172-401	+	6.83	23.09	13.64	46.56	6.85	1.79	0.96
5374-A5403-172-401	+	8.32	20.32	11.94	47.05	9.36	1.69	1.02
average		7.06	22.53	13.91	46.37	7.08	1.77	0.99
5374-A5403-36-353	+	6.18	30.73	11.36	41.3	7.3	1.92	0.89
5374-A5403-36-353	+	6.42	30.82	11.14	41.03	7.53	1.85	0.85
5374-A5403-36-353	+	6.43	30.03	11.61	40.84	8.12	1.84	0.84
5374-A5403-36-353	+	6.66	29.27	13.98	40.82	6.26	1.77	0.83
5374-A5403-36-353	+	6.15	30.32	13.67	40.76	5.95	1.92	0.89
average		6.37	30.23	12.35	40.95	7.03	1.86	0.86
5374-A5403-36-489	+	6.57	34.87	10.56	37.1	7.23	2.34	1.09
5374-A5403-36-489	+	6.25	37.1	8.43	37.65	7.05	2.33	1
5374-A5403-36-489	+	6.36	36.22	10.68	36.18	6.88	2.39	1.08
5374-A5403-36-489	+	6.29	36.28	8.69	38.06	7.08	2.33	1.04
5374-A5403-36-489	+	6.26	36.6	8.33	37.79	7.25	2.44	1.11
average		6.35	36.21	9.34	37.36	7.10	2.37	1.06
Control								
5374-A5403-36-341	-	10.7	6	24.46	50.9	6.29	0.53	0.61
5374-A5403-36-341	-	11.2	4.92	20.68	54.35	7.37	0.47	0.57
5374-A5403-36-341	-	11.27	4.27	23.71	52.72	6.55	0.43	0.53
5374-A5403-36-341	-	11.33	4.78	20.4	54.38	7.58	0.46	0.55
5374-A5403-36-341	-	11.55	4.52	18.59	55.07	8.69	0.46	0.56
null segregant Ave		11.21	4.90	21.57	53.48	7.30	0.47	0.56

The results of the fatty acid compositional analysis for transformed 5378 soybean plants are shown in Table 3. Seeds of soybean plants transformed to express *Garm FatA1* from the 7S promoter produced increased levels of stearate over those levels observed in seeds of 5 nontransformed control plants. In the seed oil of some T1 5378 transgenic soybean, stearate levels of as high as approximately 53% of the fatty acids were obtained, while levels of approximately 4% were observed in nontransformed control plants.

Table 3

STRAIN ID	GUS	%16:0	%18:0	%18:1	%18:2	%18:3	%20:0	%22:0
5378-A5403-28	+	6.27	41.81	8.64	34.22	5.13	2.62	1
5378-A5403-28	+	6.3	42.63	10.15	32.76	4.16	2.55	0.87
5378-A5403-28	+	6.48	43.11	7.47	33.72	5.3	2.61	1.04
5378-A5403-28	+	6.48	43.12	9.32	32.97	4.14	2.64	1.02
AVERAGE		6.38	42.67	8.90	33.42	4.68	2.61	0.98
5378-A5403-48	+	8.19	25.51	12.37	44.21	6.85	1.62	0.88
5378-A5403-48	+	7.74	33.77	12.13	36.33	6.41	2.23	1.05
5378-A5403-48	+	7.42	40.06	9.82	30.97	7.26	2.84	1.23
5378-A5403-48	+	7.89	45.26	5.73	30.23	6.8	2.71	1.06
5378-A5403-48	+	7.04	47.2	5.69	29.58	6.13	2.86	1.2
AVERAGE		7.66	38.36	9.15	34.26	6.69	2.45	1.08
5378-A5403-59	+	9.96	47.1	9.17	22.11	4.12	4.78	2.3
5378-A5403-59	+	7.06	47.3	4.44	29.02	7.17	3.31	1.44
5378-A5403-59	+	11.72	50.5	4.69	20.86	5.35	4.34	1.93
5378-A5403-59	+	7.55	51.95	4.99	24.53	5.02	4	1.72
AVERAGE		9.07	49.21	5.82	24.13	5.42	4.11	1.85
5378-A5403-60	+	7.7	35.2	6.09	37.85	8.77	2.74	1.34
5378-A5403-60	+	7.19	35.53	5.86	38.36	8.57	2.79	1.41
5378-A5403-60	+	7.27	36.4	5.51	37.78	8.61	2.78	1.35
5378-A5403-60	+	9.01	52.21	1.71	23.14	7.62	4.41	1.59
5378-A5403-60	+	9.83	52.94	1.77	21.85	7.59	4.28	1.42
AVERAGE		8.2	42.46	4.19	31.80	8.23	3.40	1.42
5378-A5403-69	+	11.67	4.67	18.46	55.85	7.93	0.43	0.47
5378-A5403-69	+	8.69	13.65	19.02	48.86	7.19	1.13	0.9
5378-A5403-69	+	8.13	18.5	14.7	48.52	7.4	1.41	0.83
5378-A5403-69	+	7.1	21.26	12.86	48.93	7.1	1.43	0.89
5378-A5403-69	+	8.04	44.02	8.09	28.04	7.34	2.96	1.16
AVERAGE		8.73	20.42	14.63	46.04	7.39	1.47	0.85
5378-A5403-103	+	9.31	40.01	8.73	30.52	7.08	2.85	1.04
5378-A5403-113	+	7.41	49.06	4.91	28.83	5.45	2.91	1.06
5378-A5403-113	+	11.01	49.44	4.88	22.53	7.15	3.24	1.1
5378-A5403-113	+	7.03	49.79	4.09	29.08	5.9	2.79	1
5378-A5403-113	+	8.32	51.04	4.29	27.06	5.1	2.91	0.92
5378-A5403-113	+	8.52	52.59	3.69	26.35	4.64	2.93	0.86
AVERAGE		8.46	50.38	4.37	26.77	5.65	2.96	0.99
Control								
A5403	-	11.62	4.3	24.26	49.84	7.47	0.48	0.57
A5403	-	12.32	4.24	21.93	52.32	7.49	0.44	0.46
A5403	-	12.64	4.25	20.49	53.42	7.81	0.43	0.51
A5403	-	12.17	4.22	21.56	52.48	8.15	0.44	0.51
A5403	-	11.68	4.32	25.68	49.67	7.06	0.48	0.54
AVERAGE		12.09	4.27	22.78	51.55	7.60	0.45	0.52

In T3 seed of selected T2 soybean lines, increases in stearate of as high as approximately 53% of the total fatty acid composition were obtained (Table 4), similar to those levels obtained from seed oil from T1 5378 soybean lines. Furthermore, similar to the 5374 soybean plants, decreases in palmitate, oleate and linoleate were observed in both the T2 and T3 seed oil. In 5 addition, increases in saturates greater than C18:0 are also obtained in both the T2 and T3 generations.

Table 4

STRAIN ID	GUS	16:0	18:0	18:1	18:2	18:3	20:0	22:0
5378-A5403-48-269	+	8.63	50.62	5.5	23.07	7.4	3.34	1.14
5378-A5403-48-269	+	8.27	53.31	4.54	23.14	6.2	3.26	1.09
5378-A5403-48-269	+	8.6	51.92	4.84	22.65	7.27	3.43	1.01
5378-A5403-48-269	+	8.62	51.62	4.39	23.2	7.43	3.46	1.06
5378-A5403-48-269	+	9.07	50.57	4.77	22.61	7.97	3.52	1.22
average		8.64	51.61	4.81	22.93	7.25	3.40	1.10
5378-A5403-113-304	+	6.99	49.4	4.23	29.37	6.12	2.73	0.98
5378-A5403-113-304	+	6.79	50.25	3.77	28.53	6.76	2.74	0.99
5378-A5403-113-304	+	6.79	50.19	3.73	28.8	6.61	2.75	0.98
5378-A5403-113-304	+	6.34	47.67	4.08	30.03	7.96	2.77	1.01
5378-A5403-113-304	+	6.81	49.89	3.93	29.16	6.43	2.63	0.92
average		6.74	49.48	3.95	29.18	6.78	2.72	0.98
5374-A5403-36-341	-	10.7	6	24.46	50.9	6.29	0.53	0.61
5374-A5403-36-341	-	11.2	4.92	20.68	54.35	7.37	0.47	0.57
5374-A5403-36-341	-	11.27	4.27	23.71	52.72	6.55	0.43	0.53
5374-A5403-36-341	-	11.33	4.78	20.4	54.38	7.58	0.46	0.55
5374-A5403-36-341	-	11.55	4.52	18.59	55.07	8.69	0.46	0.56
null segregant Ave		11.21	4.90	21.57	53.48	7.30	0.47	0.56

10 The above results demonstrate that by expression of an acyl-ACP thioesterase with substantial activity towards 18:0 acyl-ACP substrates, and capable of producing C18:0 in seed tissue of soybean plants, it is now possible to increase the levels of stearate in the seed oil of soybean.

15 **Example 4:** Composition of high stearate soybean oil.

In this particular case, soybean variety Hartz H4152 (also known as HS-2) was developed with a unique fatty acid composition of about 24% or above stearate and below about 3%, preferably about 2.5% linolenate. H4152 was derived from the cross between a soybean line with high stearate content (H90-127-113, also known as HS-1) and a line with low linolenic content (N85-2176). H90-127-113 is a Hartz variety derived from a cross between Hartz variety H5668 and soybean line A6. A6 is a soybean mutant with high stearate seed content (28.1%) released in 1981 by Iowa State University (Hammond, E.G. and W.R. Fehr. 1983. Registration of A6 germplasm line of soybean. *Crop Sci.* 23: 192-193). The high stearate content in line A6 has been determined to be conditioned by a single recessive gene, *fas-a* (Graef, G.L., W.R. Fehr, 10 and E.G. Hammond. 1985. Inheritance of three stearic acid mutants of soybean. *Crop Sci.* 25: 1076-1079). N85-2176 is a release from North Carolina State University selected for its low linolenate seed content.

The F_1 seeds from the cross between H90-127-113 and N85-2176 were grown in the greenhouse in November and December. In February, small portions of the F_2 seeds opposite 15 the embryo were removed and analyzed in the laboratories of Hartz Seed Co. for fatty acid composition using a gas chromatography. F_2 seed number 27 was selected for its high stearate and low linolenate levels and was grown in the greenhouse. The F_3 seed from plant #27 were planted in the field at Stuttgart, AR in the following summer. Ten agronomically desirable F_3 plants with desirable fatty acid composition were selected, and F_4 progeny rows from those 20 plants were planted at Stuttgart in the summer. Twenty agronomically desirable, uniform single plants were selected from row number 4. The F_5 progeny of those 20 plants was planted in single rows in a winter nursery in Santa Isabel, Puerto Rico. Ten uniform, single rows were harvested and bulked. The resulting seed was grown in a 0.2 acre breeder increase at Stuttgart, AR during the following summer, forming the foundation for Hartz variety H4152.

25

Table 5: Fatty acid distributions of soybean oil

<u>Soybean oil</u>	<u>Palmitic</u> <u>C16:0</u>	<u>Stearic</u> <u>C18:0</u>	<u>Oleic</u> <u>C18:1</u>	<u>Linoleic</u> <u>C18:2</u>	<u>Linolenic</u> <u>C18:3</u>
Common	11	4	23	53	8
High stearate (H4152)	10	21	22	41	3

The composition of the high stearate soybean oil is unique in that it has low linoleic and linolenic and high oleic and stearic fatty acids. This improves the stability of the oil to oxidative

degradation and also changes the triglyceride composition, resulting in the formation of compounds that have a higher melting point than those found in common soybean oil. The melting point of high stearate soybean oil is below room temperature, but solid fats are present that crystallize when the oil is stored at room temperature.

5

Example 5: Stability of high stearate soybean oil.

Table 6: Stability assays of soybean oil

Stability criteria	Common soybean oil	High stearate soybean oil
Inherent stability	7.0	3.9
Calculated iodine value	132	101
Active oxygen method	8-10	40

Inherent stability is a calculated relative reactivity with oxygen, with higher values denoting a greater predisposition to oxidation (M. Erickson and N. Frey, *Food Technology*, **50**: 63-68 (1994)). Iodine values represent calculated reactivities with elemental iodine, with higher values indicating greater reactivities(Official and Tentative Methods, American Oil Chemists' Society, Cd 1b-87, Champaign, IL). The active oxygen method assay simulates thermal breakdown encountered during cooking, with higher values representing greater thermal stability (Official and Tentative Methods, American Oil Chemists' Society, Cd 12-57(93), Champaign, IL). The increase in saturated and monounsaturated fatty acids, and the decrease in polyunsaturated fatty acids in high stearate soybean oil results in a greater thermal stability when compared to common soybean oil. The greater thermal stability of high stearate soybean oil when compared to common soybean oil agrees with predictions based upon the calculated inherent stabilities and iodine values.

Example 6: Solids profile of soybean oils

Table 7: Solids present at various temperatures

Oil type	Solids present at temperature				
	50°F	70°F	80°F	92°F	104°F
Common soybean oil	-	-	-	-	-
High stearate (H4152)	9.8	-	-	-	-
Interestesterified high stearate	7.0	3.5	3.0	2.0	0.7

Solids were evaluated by the solids fat index using dilatometry. Values represent percent solids in a sample at the given temperature. Interestesterified high stearate oil was prepared by

interesterification according to the method of Erickson (Practical Handbook of Soybean Processing and Utilization, American Oil Chemists' Society Press, Champaign IL, 1995). In contrast, soybean oil A6 having high stearic, normal levels of palmitic and linolenic, and low levels of oleic and linoleic acids, has no solids present at 24.7°C and higher temperatures.

Example 7: Application of high stearate soybeans in soy based foods.

A. To prepare tofu and soymilk, 50 grams of high stearate soybeans were soaked in 150 grams water overnight and drained. Soybeans were rinsed with water. 185 grams of boiling water was added, and the mixture pureed. 315 grams of water were added, and the mixture 5 heated to 100°C for 10 minutes. Okara (filtered solids) was extracted using the Juiceman Junior machine (Salton Maxim, Mt. Prospect, IL) to dehull the beans. The hot soymilk should be approximately 8% solids. For firm tofu, 1.5 grams glucono- δ -lactone (Aldrich, Milwaukee, WI) was added. The liquid was allowed to coagulate for 15 to 20 minutes at 90°C with light stirring. The liquid was allowed to cool and form tofu.

10 B. To prepare soymilk, 150 grams of high stearate soybeans were soaked in 500 mL water overnight and drained. Soaked soybeans were rinsed with water. The beans were equally divided into two portions. Each portion was ground with 400 mL water in an Oster blender (Sunbeam, Delray Beach, FL) at the highest speed for 1.5 minutes. The combined slurry from the two portions was manually filtered through cloth. The solid residue was discarded. The 15 filtrate was heated to 95°C for 10 minutes to prepare soymilk. To prepare tofu, the hot soymilk was cooled to about 75°C, and 5 grams of either calcium sulfate or glucono- δ -lactone was added. The mixture was allowed to stand for 30 minutes to form curd, which became silken tofu. To prepare firm tofu, the hot curd was broken, placed in a mold, and pressed to release the whey.

20 Tofu prepared from high stearate oil soybeans had a consistency that was firmer and more creamy than tofu prepared from standard control soybeans.

Example 8: Applications of full fat soy flour in baked foods.

High stearate soybeans were processed into full fat soy flour using the standard industry protocol (Practical Handbook of Soybean Processing and Utilization, American Oil Chemists' Society Press, Champaign IL, 1995). The flour can be added to baked products at high levels

(15-20%) to increase the protein content without affecting the texture of the baked products. Flour obtained from high stearate soybeans can be used in an array of food products including candies, gravies, sauces, frozen desserts, pastas, meat products, and baked goods.

Example 9: Margarine formulation using high stearate soybean oil

5

Table 8: Margarine composition

<u>Ingredient</u>	<u>Weight percent</u>
Water	16.85
Whey protein	0.4
Salt	1.9
Lecithin	0.4
Monoglyceride (Super G7, AC Humko Co)	0.45
Sodium benzoate	0.001
Interestesterified high stearate oil	80.0
Flavor, color to 100% (I-1435, Fries & Fries)	

The oil was heated to 65°C in a microwave oven. Flavors were withheld and added to the oil phase immediately before the oil phase was combined with the remaining components which had been heated to 50°C in a microwave oven. The two phases were combined and mixed for 20 minutes at 2000 rpm in a Dispermat unit (VMA-Getzmann, Germany) maintained at 60°C. The margarine was filled into one pound tubs and placed at 40°C for crystallization. 10 The margarine product was easily spreadable when removed from refrigeration after one day, and after long term storage of four weeks. The margarine exhibited good room temperature stability as well as excellent flavor release and structure.

Example 10: Formulation of all-purpose shortenings with interestesterified high stearate oil.

15 Commercial shortenings, such as CRISCO (Proctor and Gamble, Cincinnati, OH), are composed of a hydrogenated soybean oil combined with a fully hydrogenated cottonseed oil component and an emulsifier such as a monoglyceride. The soybean oil basestocks generally have a trans fatty acid content of greater than 15% (w/w), and frequently greater than 25% (w/w). The interestesterified high stearate soybean oil has sufficient solids such that when 20 combined with the fully hydrogenated cottonseed oil and monoglyceride, it gives a texture and consistency similar to the CRISCO product upon vocation (rapid chilling and working of fat, Weiss, T.J., Food oils and Their Uses, Avi Publishing Co., Westport, CT, 1983) and

crystallization. Nitrogen is added to the formulation to modify the final density and solidity of the product. A 15% overrun of gas corresponds to a 15% reduction in density of the shortening in comparison to the density before addition of nitrogen.

Table 9: Shortening formulation

<u>Component</u>	<u>Percent by mass</u>
Interestesterified high stearate oil	89.2
Cottonseed oil (5 iodine value max.)	7.8
Monoglyceride (Super G7, AC Humko Co)	3.0
Nitrogen gas	15% overrun

5 **Example 11:** Shelf life testing

Non-hydrogenated oils are preferred over partially hydrogenated oils due to costs and improved acceptance by the consumer. However, the short shelf stability of non-hydrogenated oils severely limit their food applications. The interestesterified high stearate soybean oil was used in a potato chip rancidity assay to determine the applicability in the preparation of fried foods.

10

Table 10: Schaal oven test

<u>Oil</u>	<u>Days to detect rancid odor</u>
Common soybean oil	2-3
All-purpose shortening (CRISCO)	6-7
Interestesterified high stearate oil (all-purpose shortening formulation)	11

15

The rancidity assay was performed using a Schaal oven test at 62°C (Warner, K. and Eskin, N.M., Methods to Assess Quality and Stability of Oils and Fat-Containing Foods, American Oil Chemists' Society Press, Champaign, IL, 1995). Interestesterified high stearate oil demonstrated a marked increase in stability, as indicated by the longer duration of time required to detect an undesirable odor.

20

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.

Claims

What is claimed is:

1. A method for increasing stearate as a component of total triglycerides found in

5 soybean seed, wherein said method comprises:

growing a soybean plant having integrated into its genome a DNA construct, said construct comprising in the 5' to 3' direction of transcription, a promoter functional in a soybean plant seed cell, a DNA sequence encoding an acyl-ACP thioesterase protein having substantial activity on C18:0 acyl-ACP substrates, and a transcription termination region functional in a

10 plant cell.

2. The method of Claim 1, wherein said encoding sequence has little or no activity on C16:0 substrates.

3. The method of Claim 1 wherein said thioesterase encoding sequence is from the genus *Garcinia*.

15 4. The method of Claim 1, wherein said DNA sequence encodes the *Garm* FatA1 thioesterase presented in Figure 1.

5. The method of Claim 1, wherein said promoter is from a gene preferentially expressed in plant seed tissue.

6. The method according to Claim 1, wherein said promoter is the napin promoter.

20 7. The method according to Claim 1, wherein said promoter is from the soybean β -conglycinin 7S subunit transcription initiation region.

8. The method of Claim 1, wherein said plant seed triglycerides comprise about 20 weight percent or greater stearate.

9. The method of Claim 1, wherein said plant seed triglycerides comprise about 33 weight percent or greater stearate.

10. The method of Claim 1, wherein said plant seed triglycerides comprise about 40 weight percent or greater stearate.

5 11. The method of Claim 1, wherein said plant seed triglycerides comprise about 50 weight percent or greater stearate.

12. A soybean seed comprising about 33 weight percent or greater stearate as a component of total fatty acids found in seed triglycerides.

13. The soybean seed according to Claim 12 comprising about 40 weight percent or 10 greater stearate as a component of said seed triglycerides.

14. The soybean seed according to Claim 13 comprising about 50 weight percent or greater stearate as a component of said seed triglycerides.

15. The soybean seed according to Claim 12 comprising about 50 percent or greater stearate as the fatty acid found at the sn-1 and sn-3 positions of seed triglycerides.

15 16. The soybean seed according to Claim 13 comprising about 60 percent or greater stearate as the fatty acid found in said sn-1 and sn-3 positions of seed triglycerides.

17. The soybean seed according to Claim 14 comprising about 75 percent or greater stearate as the fatty acid found in said sn-1 and sn-3 positions of seed triglycerides.

18. Soybean seed according to Claim 15, and comprising oleic acid as the 20 predominant fatty acid at the sn-2 position of seed triglycerides.

19. Seed oil obtained from seed of Claim 12.

20. A shortening comprising the seed oil of Claim 19.

21. A soybean plant having seed comprising about 50 weight percent or greater saturated fatty as a component total fatty acids found in seed triglycerides.

22. The soybean plant according to Claim 21 wherein said saturated fatty acid component is about 60 weight percent or greater.

5 23. The soybean plant according to Claim 22 wherein said saturated fatty acid component is about 65 weight percent or greater.

24. A soybean plant having seed comprising about 20 weight percent or greater stearate and about 15 weight percent or less oleate as components of total fatty acids found in seed triglycerides.

10 25. The soybean plant according to Claim 24 wherein said stearate content is greater than about 33% of the total fatty acid composition.

26. A vegetable oil-based edible spread comprising a high stearate soybean oil.

27. The vegetable oil-based edible spread of claim 26, wherein said soybean oil comprises a stearate level of about 20 weight percent or greater.

15 28. The vegetable oil-based edible spread of Claim 26, wherein said soybean oil is the seed oil of the mutant soybean variety HS2.

29. The vegetable oil-based edible spread of Claim 26, wherein said soybean oil is the seed oil of Claim 19.

30. A frying oil comprising a high stearate soybean oil.

20 31. The frying oil of claim 30, wherein said soybean oil comprises a stearate level of about 20 weight percent or greater.

32. The frying oil of Claim 30, wherein said soybean oil is the seed oil of the mutant soybean variety HS2.

33. The frying oil of Claim 30, wherein said soybean oil is the seed oil of Claim 19.

34. A cocoa butter substitute comprising a high stearate soybean oil.

5 35. The cocoa butter substitute of claim 34, wherein said soybean oil comprises a stearate level of about 20 weight percent or greater.

36. The cocoa butter substitute of Claim 34, wherein said soybean oil is the seed oil of the mutant soybean variety HS2.

10 37. The cocoa butter substitute of Claim 34, wherein said soybean oil is the seed oil of Claim 19.

38. A cocoa butter equivalent comprising a high stearate soybean oil.

39. The cocoa butter equivalent of claim 38, wherein said soybean oil comprises a stearate level of about 20 weight percent or greater.

15 40. The cocoa butter equivalent of Claim 38, wherein said soybean oil is the seed oil of the mutant soybean variety HS2.

41. The cocoa butter equivalent of Claim 38, wherein said soybean oil is the seed oil of Claim 19.

42. A soymilk produced from soybean seed having a high stearate oil.

20 43. The soymilk of claim 42, wherein said oil comprises a stearate level of about 20 weight percent or greater.

44. The soymilk of Claim 42, wherein said oil is the seed oil of the mutant soybean variety HS2.

45. The soymilk of Claim 42, wherein said oil is the seed oil of Claim 19.

46. A tofu product produced from soybean seed having a high stearate oil.

5 47. The tofu product of claim 46, wherein said oil comprises a stearate level of about 20 weight percent or greater.

48. The tofu product of Claim 46, wherein said oil is the seed oil of the mutant soybean variety HS2.

49. The tofu product of Claim 46, wherein said oil is the seed oil of Claim 19.

10 50. A flour produced from soybean seed having a high stearate oil.

51. The flour of claim 50, wherein said oil comprises a stearate level of about 20 weight percent or greater.

52. The flour of Claim 50, wherein said oil is the seed oil of the mutant soybean variety HS2.

15 53. The flour of Claim 50, wherein said oil is the seed oil of Claim 19.

CCAAG	ATG	TTG	AAG	CTC	TCT	TCC	CGA	AGC	CCA	TTG	GCC	CGC	ATT	CCC	50		
Met	Leu	Leu	Ser	Ser	Ser	Ser	Arg	Ser	Pro	Leu	Ala	Arg	Ile	Pro			
															15		
5																	
ACC	CGG	CCC	AGG	CCC	AAC	TCC	ATT	CCT	CCC	CGG	ATA	ATT	GTG	GTT	TCC	98	
Thr	Arg	Pro	Arg	Pro	Asn	Ser	Ile	Pro	Pro	Arg	Ile	Ile	Val	Val	Ser		
															30		
20																	
TCC	TCA	TCC	AGC	AAG	GTT	AAT	CCA	C _T TC	AAA	ACA	GAG	GCG	GTG	GTT	TCT	146	
Ser	Ser	Ser	Ser	Ser	lys	lys	Val	Asn	Pro	Leu	Lys	Thr	Glu	Ala	Val	Ser	
															45		
35																	
TCG	GGG	CTG	GCT	GAC	CGG	C _T TC	CGG	CTG	GGC	AGC	TTG	ACC	GAG	GAC	GGG	194	
Ser	Gly	Leu	Ala	Asp	Arg	Leu	Arg	Leu	Gly	Ser	Leu	Arg	Cys	Tyr	Glu		
															60		
50																	
CTT	TCG	TAT	AAG	GAG	AAG	T _T TC	ATA	GTG	AGA	TGC	TAT	GAG	GTT	GGG	ATT	242	
Leu	Ser	Tyr	Lys	Glu	Lys	Phe	Ile	Val	Arg	Cys	Tyr	Glu	Val	Gly	Ile		
															75		
65																	
AAC	AAG	ACC	GCT	ACT	GTT	GAG	ACT	ATT	GCC	AAC	CTC	TTG	CAG	GAG	GTT	290	
Asn	Lys	Thr	Ala	Thr	Val	Glu	Thr	Ile	Ala	Asn	Leu	Gln	Glu	Val			
															95		
80																	
85																	
90																	
95																	

FIGURE 1

GGA	TGC	AAT	CAC	GCC	CAA	AGC	GTT	GGA	TAT	TCG	ACG	GGT	GGG	TTT	TCG	338
Gly	Cys	Asn	Asn	His	Ala	Gln	Ser	Val	Gly	Tyr	Ser	Thr	Gly	Gly	Phe	Ser
																110
																105
ACA	ACC	CCT	ACC	ATG	AGA	AAA	TTC	CGT	ATA	TGG	GTT	ACT	GCT	CGC		386
Thr	Thr	Pro	Thr	Met	Arg	Lys	Leu	Arg	Leu	Tyr	Leu	Trp	Val	Thr	Ala	Arg
																125
ATG	CAC	ATC	GAA	ATC	TAC	AAA	TAT	CCA	GCT	TGG	AGT	GAT	GTG	GTG	GAA	434
Met	His	Ile	Glu	Ile	Tyr	Lys	Tyr	Pro	Ala	Trp	Ser	Asp	Val	Val	Glu	
																140
ATA	GAG	TCG	TGG	GGC	CAG	GGT	GAA	GGA	AAA	ATC	GGA	ACC	AGA	CGT	GAT	482
Ile	Glu	Ser	Trp	Gly	Gln	Gly	Glu	Gly	Lys	Ile	Gly	Thr	Arg	Arg	Asp	
																145
TGG	ATT	CTG	AGA	GAC	TAT	GCC	ACT	GGT	CAA	GTT	ATT	GGC	CGA	GCT	ACT	530
Trp	Ile	Leu	Arg	Asp	Tyr	Ala	Thr	Gly	Gln	Val	Ile	Gly	Arg	Ala	Thr	
																160
AGC	AAG	TGG	GTA	ATG	AAC	CAA	GAC	ACC	AGG	CGA	CTT	CAA	AAA	GTC		578
Ser	Lys	Trp	Val	Met	Met	Asn	Gln	Asp	Thr	Arg	Arg	Leu	Gln	Lys	Val	
																180
GAT	GTT	GAT	GTT	CGT	GAT	GAG	TAC	TTC	GTT	CAC	TGT	CCA	AGA	GAA	CTC	626
Asp	Val	Asp	Val	Asp	Arg	Asp	Glu	Tyr	Leu	Val	His	Cys	Pro	Arg	Glu	Leu
																195
																200

AGA	TTG	GCA	TTT	CCA	GAG	GAA	AAT	AAT	AGC	AGC	TTG	AAG	AAA	ATT	TCA	674
Arg	Leu	Ala	Phe	Pro	Glu	Glu	Asn	Asn	Ser	Ser	Leu	Lys	Lys	Ile	Ser	
	210															210
AAA	CTT	GAA	GAT	CCT	TCT	CAA	TAT	TCG	AAG	CTG	GGG	CTT	GTG	CCT	AGA	722
Lys	Leu	Glu	Asp	Pro	Ser	Gln	Tyr	Ser	Lys	Leu	Gly	Leu	Val	Pro	Arg	
	225															225
AGA	GCA	GAT	CTG	GAC	ATG	AAT	CAA	CAT	GT ^T	AAT	AAT	GTC	ACC	TAT	ATT	770
Arg	Ala	Asp	Leu	Asp	Met	Asn	Gln	His	Val	Asn	Asn	Val	Thr	Tyr	Ile	
	240															240
GGA	TGG	GTG	TTG	GAG	AGC	ATG	CCT	CAA	GAA	ATC	ATT	GAT	ACC	CAT	GAA	818
Gly	Trp	Val	Leu	Glu	Ser	Met	Pro	Gln	Glu	Ile	Ile	Asp	Thr	His	Glu	
	260															260
CTG	CAA	ACC	ATA	ACA	TTA	GAC	TAC	AGA	CGG	GAA	TGC	CAA	CAT	GAT	GAT	866
Leu	Gln	Thr	Ile	Thr	Leu	Asp	Tyr	Arg	Arg	Glu	Cys	Gln	His	Asp	Asp	
	275															275
GTG	GTT	GAT	TCC	TTG	ACT	AGT	CCA	GAG	CCT	TCT	GAA	GAT	GCT	GAA	GCA	914
Val	Val	Asp	Ser	Leu	Thr	Ser	Pro	Glu	Pro	Ser	Glu	Asp	Ala	Glu	Ala	
	290															290
GT ^T	TTC	AAC	CAT	AAT	GGA	ACA	AAT	GGG	TCT	GCA	AAT	GTG	AGC	GCC	AAC	962
Val	Phe	Asn	His	Asn	Gly	Thr	Asn	Gly	Ser	Ala	Asn	Val	Ser	Ala	Asn	
	305															305
	310															310
	315															315

GAC	CAT	GGA	TGC	CGC	AAC	TTT	CTG	CAT	CTA	AGA	TTG	TCG	GGC	AAT	1010	
Asp	His	Gly	Cys	Arg	Asn	Phe	Leu	His	Leu	Leu	Arg	Leu	Ser	Gly	Asn	335
320															335	
GGA	CTT	GAA	ATC	AAC	CGT	GGT	CGT	ACT	GAG	TGG	AGA	AAG	AAA	CCT	ACA	1058
Gly	Leu	Glu	Ile	Asn	Arg	Gly	Arg	Thr	Glu	Trp	Arg	Lys	Lys	Pro	Thr	350
															345	
AGA	TGAGGCAATA	AAGTACATTAA	TGTACTTTAT	CGTTGCTTTA	GCCGGCTTCT											1111
Arg																
GGATGGTGT	ATTCCTTCTGC	TTCTTTCTCT	TCCTTTTTGT	TTTCCCTAGGG	TATCCTTCGCG											1171
TTCCTGCCTG	TAAGAGTATT	ATGTTTTCCG	TTTGCCCTGTA	AGTTGTAAT	TTGTCGAGGA											1231
ACTCGAGTCA	TTGGTTTGAAAT	CGAGGGATGGT	GAGAAGTGTAA	CTTGTGTTGTT	GTATTCACATT											1291
CTTCCCTGAT																1300

FIGURE 1

INTERNATIONAL SEARCH REPORT

Interna	Application No
PCT/US 99/17109	

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 7	C12N15/82	C12N15/55	C12N9/16	A01H5/00	C11B1/00
	A23C11/10	A23D9/00	A23C20/02	A21D2/16	

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H C12B A23C A23D A21D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, L	WO 97 12047 A (LASSNER MICHAEL W ;CALGENE INC (US); KNAUF VIC C (US); KRIDL JEAN) 3 April 1997 (1997-04-03)	1-6, 8-10, 12, 13, 15-21, 26, 27, 29-31, 33-35, 37-39, 41-43, 45-47, 49-51, 53
Y	abstract; claims 1-8, 12-20; figures 4, 5; examples 1-4 page 1 -page 17 --- -/-	7

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/17109

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	WO 92 11373 A (DU PONT) 9 July 1992 (1992-07-09) claims 1-14; examples 1,7,10 page 1 -page 4 page 8, line 24 -page 12, line 10 page 25, line 27 -page 30, line 12 ----	7
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